IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kaare M. GAUTVIK et al.

Title: *Production of Human Parathyroid Hormone From Microorganisms*

Appl. No.: 08/340,664

Filing Date: November 16, 1994

Examiner: L. Spector

Art Unit: 1647

DECLARATION OF KAARE M. GAUTVIK, M.D.

PURSUANT TO 37 C.F.R. § 1.132

Mail Stop NON-FEE AMENDMENT
Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, KAARE M. GAUTVIK, declare as follows:

1. I am a co-inventor of the above-captioned application.

2. I am a citizen of Norway residing at Bregnevn 3, 0875 Oslo, Norway. I am fluent in English. My curriculum vitae is attached hereto as exhibit A.

3. The standard hPTH(1-84) referred to on page 7, line 19, of the specification is synthetic hPTH obtained from chemical supply companies, including Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem.

4. I participated in the experiment detailed below to study the purity of the synthetic hPTH obtained from Peptide Institute Protein Research Foundation, Penninsula

Laboratories, Sigma, and Bachem as compared to recombinant hPTH produced according to the claimed invention. The experiment was performed on November 30, 1989 and the commercial hPTH preparations were purchased from 1986-1989.

Experimental Method

5. 0.2 μg of hPTH(1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem, and from hPTH(1-84) produced according to the claimed invention, was analyzed on an SDS-PAGE gel. The protein bands were stained by silver nitrate. Silver nitrate is the most sensitive stain to disclose impurities. The hPTH(1-84) was loaded onto the gel as follows. Where available, lot numbers, catalog numbers, and date of arrival to the laboratory are provided.

Lane 1: BRL, low molecular weight standard

Lane 2: Peptide Institute Protein Research Foundation, lot no 350306, arrived July 1986

Lane 3: Penninsula Laboratories, arrived July 27th 1986

Lane 4: Sigma P-7036, arrived before October 1st, 1987

Lane 5: Bachem, lot no ZF567, arrived July 1989

Lane 6: Bachem, lot no 734B, arrived February 1989

Lane 7: *E.coli* PTH (recombinant) (produced according to claimed invention)

Lane 8: Yeast PTH (recombinant) (produced according to claimed invention)

Lane 9: Yeast (Q26) PTH (recombinant) (produced according to claimed invention)

Lane 10: BRL, low molecular weight standard

Results

6. A photograph of the SDS-PAGE gel obtained from the above-described experiment is provided as Exhibit B. Since the lanes of the gel were loaded with the same amount of hPTH(1-84), 0.2 μg, according to the manufacturer's description, the intensity/size of the bands should be the same. However, as shown in the SDS-PAGE gel picture attached as Exhibit B, the intensity/size of the bands is not the same for the hPTH(1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem (lanes 2-6), as compared to the recombinant hPTH produced according to the claimed invention (lanes 7-9). Further, impurities (shadows appearing above or below the main band) are apparent in the lanes containing hPTH(1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem, but are virtually absent in the lanes containing recombinant hPTH produced according to the claimed invention. A more detailed analysis of the results of the gel is provided below.

7. Lane 2: The molecular weight of the main band is higher than the expected molecular weight for hPTH(1-84). Impurities are seen on the photograph above and below the major band. The material in lane 2 contains less hPTH(1-84) than the manufacturer's stated quantity.

8. Lane 3: The molecular weight of the main band is higher than the expected molecular weight for hPTH(1-84). Impurities are seen on the photograph above and below the major band. The material in lane 3 contains less hPTH(1-84) than the manufacturer's stated quantity.

9. Lane 4: Low molecular weight impurities are present. The material in lane 4 contains less hPTH(1-84) than the manufacturer's stated quantity. The impurities lead to false weight estimations.

10. Lane 5: Low molecular weight impurities are present. The material in lane 5 contains less hPTH(1-84) than the manufacturer's stated quantity. The impurities lead to false weight estimations.

11. Lane 6: Low molecular weight impurities are present. The material in lane 6 contains less hPTH(1-84) than the manufacturer's stated quantity. The impurities lead to false weight estimations.

12. Lane 7: The hPTH(1-84) is more than 95% pure hPTH(1-84) of the correct molecular weight. There is virtually no material above or below the major protein band.

13. Lane 8: The hPTH(1-84) is more than 95% pure hPTH(1-84) of the correct molecular weight. There is virtually no material above or below the major protein band.

14. Lane 9: QPTH moves a little faster than hPTH(1-84) because of one amino acid residue substitution, and there is no sign of impurities

15. Although the synthetic hPTH (1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem was impure, this synthetic hPTH (1-84) was used as a hPTH (1-84) standard in my lab. The impure hPTH (1-84) standard was used to confirm in early experiments the identity and characteristics of hPTH (1-84) produced according to the claimed invention. The impurities present in the synthetic hPTH (1-84) standard did not as such, affect the usefulness of the synthetic hPTH (1-84) as a qualitative "standard."

16. As a consequence of impurities and incorrect amounts provided by the commercial producers, the specific biological activity of even their best preparation was found to be about 30% below that of the recombinant hormone. (*See,* e.g., exhibit C, Reppe, S., et al., 1991, JBC, 22:14198-14201, Figure 5).

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 07.27.04

KAARE M. GAUTVIK, M.D.

June 2003

CURRICULUM VITAE
RELEVANT PUBLICATIONS
KAARE M. GAUTVIK,M.D.,PHD, CHIEF CONSULTANT

Personal and marital status:

Name:	**Kaare M. Gautvik**
Home address:	Bregnevn. 3, 0875 Oslo, Norway
Business address:	Institute of Medical Biochemistry, University of Oslo, P.O.Box 1112 Blindern, 0317 Oslo, Norway
Telephones:	47-22851055 (work); 47-22235137 (home)
Date and place of birth:	11th of December 1939 in Oslo.
Social Security:	No.: 111239.39311
Married to:	Vigdis Teig Gautvik, date of birth: 24th of March 1947
Children:	Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964 Silja Marie Sakrisvold Gautvik, date of birth: 31th of March 1973,Ole Martin Teig Gautvik, date of birth: 21th of January 1982

Education and Clinical Specialities:

1. August 1958-June 64, Medical School at the University of Oslo.
2. 1967-69, Courses in mathematics involving geometry, statistics and mathematical analysis.
3. May 1970, Disputation for the medical doctor degree at the University of Oslo.
4. 1985, Specialist in clinical chemistry, and physiology and nuclear medicine.
5. 1986, Specialist in occupational health medicine.

Employment:

1. June 1964-June 1965, working at Tromsø University Hospital at medical and surgical departments.
2. July 1965 until December 1965, working as a general practioner in Sjøvegan, Troms.
3. One year military service as a major in The Norwegian Air Force, working mainly at the Norwegian Institute for Aviation and Space Medicine.
4. From 1967, position as post-doctoral researcher at The Institute of Physiology, University of Oslo.
5. From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.
6. Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973-89.
7. From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.
8. From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
   (At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).
9. From Jnuary 2002 employment as senior consultant at Department of Clinical Chemistry, Labaratory Division, Ulleval University Hospital and professor II at the University of Oslo.

Post-doctoral training abroad:

1. For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.
2. From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.
3. 1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.
4. 1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.
5. 1997, 3 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

Teaching responsibility:

1. One year teaching in aviation medicine for medical personnel and pilots.
2. I have given lectures and courses for medical students in following subjects:
   Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology.
   From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.
3. Organized interfaculty advanced courses within molecular endocrinology.
4. Lectures have been given in the following subjects at post-doctoral courses:
   Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).
5. Invited lectures: Several places in the U.S., in Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.

6. Chief organizer of post graduate scientific courses for the Medical Faculty at University of Oslo, 1986-1991.
7. Organizer of international scientific meetings within the frame of the following societies:
   Acta Endocrinologica (European International Endocrine Society), The Scandinavian Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.
8. Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.
9. Invited as Symposium Lecturer at international meetings in physiology and endocrinology and molecular biology as exemplified below:

Examples of specially invited symposium lectures:

1. February, 1990: "Production of recombinant human parathyroid hormone in E.coli and Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M. Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.
2. June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.
3. July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.
4. July, 1990: Symposium lecture: "Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held by cand.scient. Sjur Reppe).
5. August, 1990: Symposium lecture: "Processing and stability of human parathyroid hormone produced in E.coli and S.cerevisiae studied by in vitro mutagenesis". Workshop/Symposium on site-directed mutagenesis and protein engineering, Tromsø, Norway.
6. December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second International Conference on Medical Microbiology and Biotechnology Towards 2000, Guangzhou, China. (Did not attend as a protest against the punishment of the students rebellion in Peking).
7. January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology Laboratory, ICI, Alderly Park, Maccelesfield SK10 4TJ, UK. Lecture entitled: "Synergistic effects of hormones and fatty acid on peroxisomal 8-oxydation, enzyme activities and mRNA levels".
8. January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell, Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning and expression of human parathyroid hormone in microorganisms".
9. Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could not attend).
10. May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biology".
11. January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA. Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of G-coupled hormones".
12. August 1993: Invited by Norwegian Society of Charted Engineers, The Blindern Conference. Lecture entitled: "Experience from industrializing basal research".
13. November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at "Graduate course in molecular endocrinology - a problem oriented approach". The lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".
14. February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH, Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in microorganisms and animal cells with special reference to signal sequence efficacy and intracellular modifications".
15. September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology, Madurai Kamaraj University, India, to held a lecture in the symposium "Gene expression systems", XVIth IUBMB, New Delhi. Lecture entitled: "Expression of human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a milk protein in transgenic mice".
16. November 1994: Invited by Professor A. Taschjian Jr., Harvard School and Public Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and functional characteristics of the human TRH receptor cDNA and mapping of the gene".
17. February 11-13, 1995: Cairns, Australia, Workshop on "Animal models in the prevention and treatment of osteopenia".
18. February 1995: Int. Meeting of Calcified tissue research, Melbourne, Australia.
19. May 1996: Dublin University Program. "How to identify patients at risk for development of ostoporosis".
20. September 1996: Lecture at Scripps Research Institute, San Diego. "Unique hypothalamic specific mRNAs expressed by molecular subtraction hybridization".
21. September 1996: Invited seminar at the Astra Research Center, Montreal. "Cloning and expression of human polypeptide hormones with biomedical potential".
22. November 1996: Invited lecturer, The Norwegian Rheumatological Society, Oslo, "PTH (parathyroidea hormone) - The biochemical foundation for treatment of osteoporosis".
23. December 1996: Invited at Nordic Conference for Medical Treatment of Osteogenesis Imperfecta, Holmen Fjordhotel, Asker, Norway. "Characteristics of bone remodelling in patients with osteogenesis imperfecta".

24. January 1997: Invited lecturer at The Salgrenska Hospital in Sweden. "Characterization and functional analysis of novel hypothalamus genes as identified by directional tag substraction".
25. 1998: Guest lecturer at Scripps Research Institute: "Hypothalamic calcium-calmodulin kinase-cloning and functional aspects".
26. February 1999: Only invited speaker from abroad at National Osteoporosis Congress in Rio de Janeiro, Brazil.
27. 2000: Lecture at NPS-Allelix company and Toronto University: "Parathyroid hormone regulated bone remodelling".
28. May 2000, Rio de Janeiro, Brazil. Member of the International Scientific Panel at the International Congress in Osteoporosis.

Honorary lectures and prizes:

1. In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize and lecture was created by Professor Torgersen, the University of Oslo, who was one of the founders of the Society for Promotion of Cancer Research in Norway. Because he contributed with personal money, the prize and lecture had his name. The title of my lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone producing cancer".
2. In 1984 I was given the international science prize called The Nordic Insulin Prize instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen. This prize is given within endocrinology and the candidate is chosen from all the countries in Northern Europe. The money was donated by the Insulin Laboratory now the company Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The prize was given for my studies regarding how hormones exerted their biological actions in target cells.
3. The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists. This is a prize which is given to a scientist selected by this society for scientific merits obtained and again it was within the field of hormone structure and action.
4. In 1987 I received a prize within biotechnology created by the Research Park at the University of Oslo, which at that time was called the Innovation Centre, University of Oslo.
5. Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska Institute, Sweden, in 1991. This was given based on my research with human parathyroid hormone in relation to its first cloning, expression and studies of actions.
6. Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995, regarding cloning of hormone genes and their characterizations. Invited by Professor A.H. Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA.
7. Given a 3 years economical "Group Research Support of 0.8 M NOK per year from 1997" after national and international project evaluations.
8. Scientific prize (Abstract award) 1997, at the Endocrine Society "Cloning and Organization of the human TRH-receptor Gene".
9. Norwegian Endocrine Society Prize (S.Reppe) for "Sox4 – a PTH regulated transcription factor in bone".
10. Endocrine Society 1998: Poster Award: Cloning and regulation of the thyroliberin receptor gene.
11. Norwegian Endocrine Society prize 1998: Hormone regulated bone remodelling.
12. American Society for Bone and Mineral Research (ASBMR); 1999 Best Poster Award: The Transcription factor Sox-4 is expressed in developing cartilage and bone cells.

Consulting appointments:

1. Senior honorary consultant for NPS Biotechnology, Salt Lake City, Colorado, USA.
2. Consultant for Karolinska Institute, Stockholm, Sweden.

Referee activity:

I am or have been working as referee for the following international journals:
Endocrinology,J. Expl. Cell Res.,Acta Physiol. Scand. (Kbh.),Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.),Eur.J.Clin. Invest.,Hormone Research,Acta Obstet. Gynecol. Scand.,Journal of Endocrinological Investigation,Eur. J. Biochem.,Experimental Cell Research,J. Biol. Chem.

Guidance for the academic doctor degree:Twenty three and 5 ongoing.
Supervision of postgraduate candidates: Presently three.
Supervision of students' main degrees: Nineteen.
Guest research workers from abroad: In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.
Member of committees for the academic doctor degree in Norway and abroad: 15.
Member of advisory international/national committees for evaluation of professor positions:14.
Honorary Societies: Member of the Norwegian National Academy of Science and Letters
Professional memberships: Norwegian Society of Biochemistry,Norwegian Society of Physiology,Norwegian Society of Endocrinology,Endocrine Society (USA),American Society for Bone and Mineral Research (USA)
Medical Faculty Responsibilities:

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Research Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's councel for evaluation of postgraduate applications from 1989-1993.
6. Committee member of the Medical Faculty's Scientific Instrument Board, 1996-.
7. Committee for Medical Research collaboration and interaction between University of Oslo and the National Hospital, 2000-.

National- and International Research Council Responsibilities:

1. Leader of Chemical Peptide Synthesis Core facility 1984-1989.

2. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
3. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
4. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
5. Member of the Premedical Institute Group Committee for Preclinical Sciences from 1989-2003.
6. Member of the International Scientific Board of Novo-Nordisk Research Committee, 1989-2001.
7. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway, 1989-present.
8. Chairman of the Research Council in the Norwegian Association for Osteoporosis, 1993-2003.
9. Leader of DNA Sequencing Core facility of the Institute of Basic Science, 1999-present.

10. Consultant and peer reviewer within Wallenberg Consortium North Technology Platforms DNA; SNP (single nucleotride polymorphism) Technologies and the Platform for Proteomics on behalf of the Board of the Wallenberg Consortium North, Stockholm, Sweden, 2001-2004.

11. Coordinator for Marie Curie Training Sites Fellowship No MCFH-200-00040 :"Oslo Doctoral Training Site for Diagnosis and Therapy of Osteoporosis"—2001-2005.

12. Coordinator of EU 6.Program STREP contract no 502941, "Molecular mechanisms of bone homeostasis" (OSTEOGENE). Eight partners in 5 countries- 2003-2006

Awards and fellowships:

1967, 3 months, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.

1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.

1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.

1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

1997 Awarded for 3 years the Norwegian Research Council's Science Prize for outstanding research.

2001 Promoted by EU to become Oslo Doctoral Training Site for Diagnosis & Therapy Of Osteoporosis, received by a group consisting of scientists from University of Oslo, IMBA and the National Hospital.

2002 Member of CNS Molecular Biology group (leader Ivar Walaas) appointed as a "Research Theme Priority" at the Medical Faculty,2002- 2007

2004 OSTEOGENE (Molecular mechanisms of bone homeostasis) project given the highest priority and the only selected for presentation within Health Region East 2004.

Other professional activities:

1. Founder of the Norwegian Association for Osteogenesis Imperfecta 1978 (Norsk Forening for Osteoporosis Imperfecta) together with Mrs. L. Myhre.
2. Founder of the Norwegian Association for Osteoporosis (Norsk Osteoporoseforening) 1993, together with Norwegian Women Public Health Association (NKS).

Patents:

I.Two U.S. patents, U.S. Patent No. 5.010 010 and No. 5.420.242 are held with international extensions in Europe, Japan, Canada, and Australia. In addition, three Divisional Applications are submitted to the U.S. Patent Office and elsewhere.These patents and patent applications in the different countries are covering specific methods related to the production, purification and characterization of PTH in microorganisms for the use in treatment of osteoporosis.

II.Inventor in patent application from Scripps Research Institute on: Novel hypothalamic mRNAs, the corresponding peptides and their functions.

Publications: *More than 200 original articles published in internationally well reputated and refereed journals. Relevant articles are cited in relation to description of the research activities :*

A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS AND RELEVANT REFERENCES

A.STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The results so far from our refinement and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method have been successful. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. We have so far described several novel peptides: hypocretin (the cause of Narcolepsy) and several very interesting peptides, e.g. novel CaM kinase (see list of references). Another CNS peptide is somatostatin-like, called cortistatin, structure similarity with somatostatin; P25 and Vat 1 , two uniquely expressed peptides in distinct regions of the brain.

1.Gautvik, K.M., de Lecea, Luis, Gautvik, V.T., Danielson, P.E., Tranque, P., Dopazo, A., Bloom, F.E. and Sutcliffe, J.G. Overview of the most prevalent hypothalamus-specific mRNAs identified by directional tag PCR subtraction.
Proc. Natl. Acad. Sci. USA (PNAS) 93: 8733-8738, 1996.

2.de Lecea, L., Criado, J.R., Prospero-Carcia, O., Gautvik, K.M., Schweitzer, P., Danielson, P.E., Dunlop, C.L.M.Siggin G.R., Henriksen, S.J. and Sutcliffe, J.G. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. Nature 381: 242-245, 1996.

3.de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X-B., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L.F.,Gautvik, V.T., Bartlett II, F.S., Frankel, W.N., Van den Pol, A.N., Bloom, F.E., Gautvik, K.M. and Sutcliffe, J.G. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA (PNAS) 95: 322-327, 1998.

B. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE BONE CELL PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described above, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual cones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumor type (Olstad et al. ,2003)

C.ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS ("Bone anabolic genes")

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of sentral importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular substraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is almost completed in a highly successful manner. We have isolated more than 40 genes which are involved in PTH anabolic action in bone, and among those we are searching for the gene(s) causing postmenopausal osteoporosis.

D. As a complementation an to the activities described above, we have embarked on defining the bone phenotype in female and male osteoporosis within the context of the EU project OSTEOGENE (see above). About 100 patients and controls will have bone biopsies which will be prepared and analysed for their global gene expression and differences at the micro- and ultrastructural level. I am the coordinator of this activity including 5 countries and where Oslo university and three hospitals(Ullevål university hospital,the National Hospital and Lovisenberg hospital are working closely together. This is a direct consequence and follow up of previous research representing patient related basic and translational science aiming to solve the mechanisms of osteoporosis, the most common disease in women of 50 yrs of age.

THE MAIN RESEARCH ACTIVITIES DURING THE LAST 8 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:

I. PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

i) Recombinant parathyroid hormone for structure activity studies in relation to bone cell activation.
ii) Study intracellular processing and trafficking of these hormones and to compare signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant parathyroid hormone in mg quantities. For this work we developed gene constructs, vector modifications, fermentation technological improvements as well as complete methods for down-stream technology. The final product is PTH identical and more than 99% pure and has shown full chemical, biochemical and biological identity with the intact hormone. These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to express secreted human parathyroid hormone in mammalian cells as well as a secretory milk product in transgenic mice. In addition, we have been the first to develop full-length PTH polypeptides with agonist and antagonist functions.

1.Høgset, A., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Jacobsen, P.B., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression of human parathyroid hormone in Escherichia coli. BBRC 166: 50-60, 1990.

2.Gabrielsen, O.S., Reppe, S., Sletten, K., Øyen, T.B., Sæther, O., Høgset, A., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression and secretion of human parathyroid hormone in Saccharomyces cerevisiae. Gene 90(2): 255-262, 1990.

3.Høgset, A., Blingsmo, O.R., Sæther, O., Gautvik, V.T., Holmgren, E., Josephson, S., Gabrielsen, O.S., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression and characterization of a recombinant human parathyroid hormone secreted by E.coli employing the staphylococcal protein A promoter and signal sequence. J. Biol. Chem. 265: 7338-7344, 1990.

In this regard we have received acceptance for an international patent on gene constructions, plasmids, the process and the down-stream technology. In the further work we have by using in vitro mutagenesis, created full length parathyroid hormone agonist which has shown to be protease resistant and have interesting biological actions regarding mobilization of calcium from bone.

Both the intact hormone as well as the agonist will represent important medical drugs for use in diagnostics as well as represent a potential drug for treatment of various diseases.

4. Reppe, S., Olstad, O.K., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Gabrielsen, O.S., Øyen, T.B., Gordeladze, J.O., Haflan, A.K., Tubb, R., Morrison, N., Tashjian, A.H. Jr., Alestrøm, P. and Gautvik, K.M. Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product. ECB, 5th European Congress on Biotechnology, Copenhagen July 8-14, 1990. (Invited).

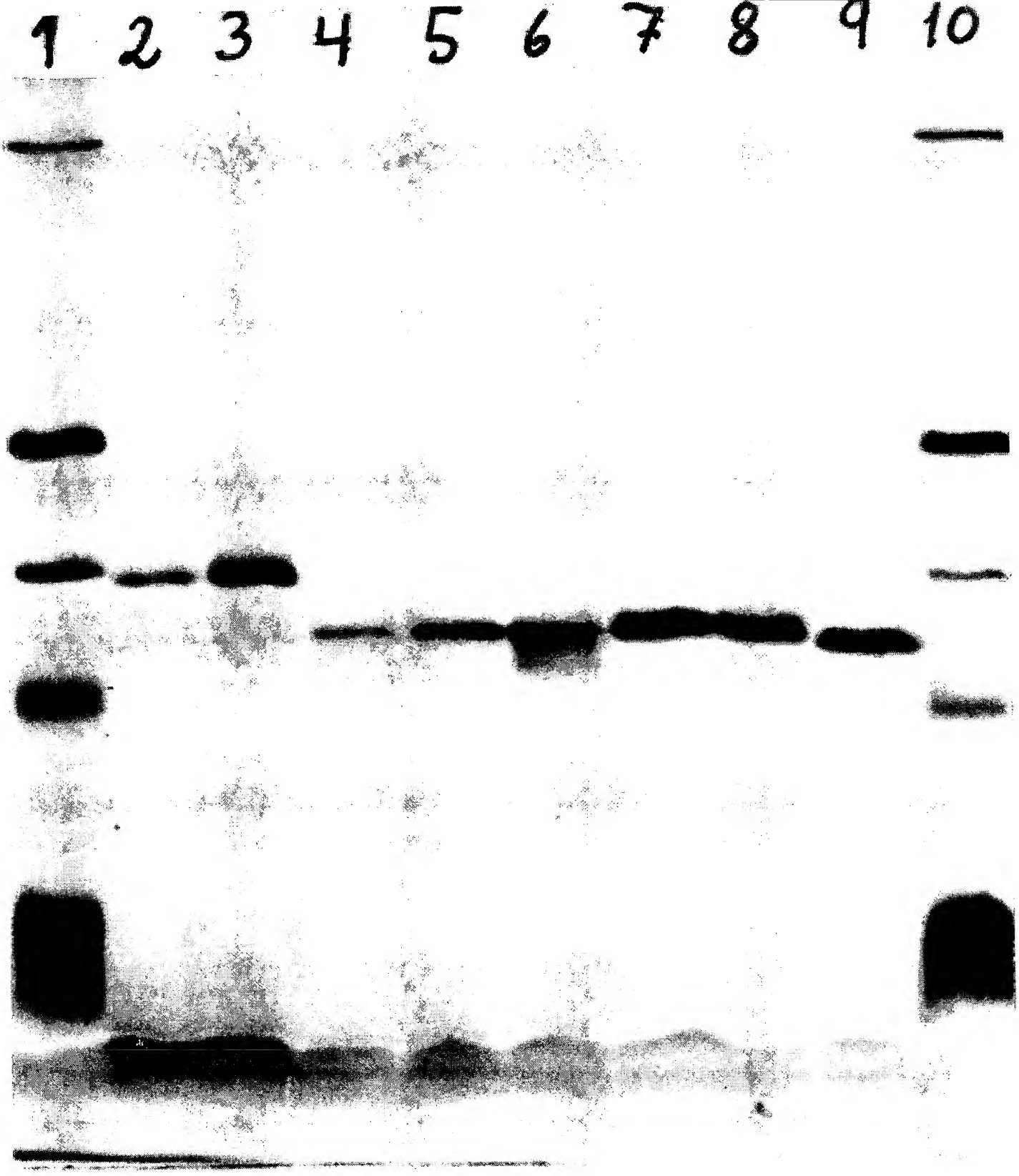
5. Reppe, S., Gabrielsen, O.S., Olstad, O.K., Morrison, N., Sæther, O., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Haflan, A.K., Voelkel, E.F., Øyen, T.B., Tashjian A.H. Jr. and Gautvik, K.M. Production of recombinant human parathyroid hormone in yeast: Synthesis, purification, and biological characterization of a Lys-26Gln site directed mutant. J. Biol. Chem. 266: 14198-14201, 1991.

6.Forsberg, G., Brobjer, M., Holmgren, E., Bergdahl, K., Persson, P., Gautvik, K.M. and Hartmanis, M. Thrombin and H64A subtilisin cleavage of fusion proteins for preparation of human recombinant parathyroid hormone. J. Protein Chem. 10(5): 517-526. 1991.

7.Olstad, O.K., Reppe, S., Gabrielsen, O.S., Hartmanis, M., Blingsmo, O.R., Gautvik, V.T., Haflan, A.K., Christensen, T.B., Øyen, T.B. and Gautvik, K.M. Isolation and characterization of two biologically active O-glycosylated forms of human parathyroid hormone produced in

Saccharomyces cerevisiae. Identification of New Motif for O-glycosylation. Eur. J. Biochem. 205: 311-319, 1992.
8.Kareem, B.N., Rokkoknes, E., Høgset, A., Holmgren, E. and Gautvik, K.M. A method for the evaluation of the efficiency of signal sequences for secretion and correct N-terminal processing of human parathyroid hormone produced in Escherichia coli. Anal. Biochem. 204: 26-33, 1992.

Recently we have expressed the first known full length antagonist for hPTH, a long sought for molecule of considerable clinical interest. The compound has a binding KD which is 2-4 times less than the natural hormone, but shows a more than 100-fold reduced biological activity.
9.Rian, E., Jemtland, R., Olstad, O.K., Gordeladze, J.O. and Gautvik, K.M. Expression of biologically active human parathyroid hormone-related protein (1-141) in Saccharomyces cerevisiae. Eur. J. Biochem. 213: 641-648, 1993.
10.Rian, E., Jemtland, R., Olstad, O.Kr., Endresen, M.J., Grasser, W.A., Thiede, M.A., Henriksen, T., Bucht, E. and Gautvik, K.M. Parathyroid hormone-related protein is produced by cultured endothelial cells; A possible role in angiogenesis. Biochem. Biophys. Res. Commun. 198(2): 740-747, 1994.
11.Kareem, N.B., Rokkones, E., Høgset, A., Holmgren, E. and Gautvik, K.M. Translocation and processing of various human parathyroid peptides in E.coli are differentially affected by protein A signal sequence mutation. Eur. J. Biochem. 220: 893-900, 1994.
12.Rokkones, E., Kareem, B.N., Olstad, O.K., Høgset, A., Schenstrøm, K., Hansson, L. and Gautvik, K.M. Expression of human parathyroid hormone in mammalian cells, Escherichia coli and Saccharomyces cerevisiae. J. Biotechnol. 33: 293-306, 1994.
13.Olstad, O.K., Morrison, N.E., Jemtland, R., Jüppner, H., Segre, G.V. and Gautvik, K.M. Differences in binding affinities of human PTH(1-84) do not alter biological potency: A comparison between chemically synthesized hormone and recombinant forms. Peptides 15: 1261-1265, 1994.
14.Rokkones, E., Fromm, S.H., Kareem, N.B., Olstad, O.K., Høgset, A., Klungeland, H., Iversen, J., Bjøro, K. and Gautvik, K.M. Human parathyroid hormone as a secretory peptide in milk of transgenic mice. J. Cell. Biochem. 59: 168-176 1995.
15.Olstad, O.K., Jemtland, R., Loseth, O.P., Bringhurst, F.R. and Gautvik, K.M. Expression and characterization of a recombinant human parathyroid hormone partial agonist with antagonistic properties: Gly-hPTH(-1+84). Peptides 16: 1031-1037, 1995.
16.Mathavan, S., Gautvik, V.T., Rokkones, E., Olstad, O.K., Kareem, B.N., Maeda, S. and Gautvik, K.M. High level production of human parathyroid hormone in Bombyx mori larvae and BmN cells using recombinant baculovirus. Gene 167: 33-39, 1995.
17.Olstad, O.K, Reppe, S., Løseth, O.P., Jemtland, R. and Gautvik, K.M. Binding and Cyclic AMP Stimulation by N-terminally Deleted Human PTHs (3-84 and 4-84) in a Homologous Ligand Receptor System. J. Bone & Mineral Res. 12: 1348-1357, 1997.
18.Jemtland, R., Lee, K. and Segre, G.V. Heterogeneity among cells that express osteoclast-associated genes in developing bone. Endocrinology 139(1): 340-349, 1998.
19.Jemtland, R., Rian, E., Olstad, O.K., Haug, E., Bruland, O.S., Bucht, E. and Gautvik KM. Two human osteoblast-like osteosarcoma cell line show distinct expression and differential regulation of parathyroid hormone-related protein. J. Bone Miner. Res. 14(6): 904-14, 1999.
20.Reppe, S., Rian, E., Jemtland, R. Olstad, O.K., Gautvik, V. and Gautvik, K.M. SOX-4 mRNA is expressed in the embryonic growth plate and regulated via the PTH / PTHrP receptor in osteoblast-like cells. J.Bone Min.Res.,15,2402-2412, 2001.
21.Olstad, O.K., Reppe, S., Rian, E., Gautvik, V., Ohlsson, C., Bruland, Ø. and Gautvik, K.M. Overview of human osteosarcoma specific or enriched mRNAs, as identified by directional tag PCR subtraction. Anticancer Research 23;2201-2216.
22.Rian, E., Reppe, S., Olstad, O.K., Gautvik, V.T., Jemtland, R., Bruland, Ø., and Gautvik,K.M. Regulation of osteoblastic gene expression by parathyorid hormone (1-84) in a human osteosarcoma cell line: Characterization by subtraction library technology. Submitted 2004.



THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1991 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 266, No. 22, Issue of August 5, pp. 14198-14201, 1991
Printed in U.S.A.

Characterization of a K26Q Site-directed Mutant of Human Parathyroid Hormone Expressed in Yeast*

(Received for publication, February 25, 1991)

Sjur Reppe‡§¶, Odd S. Gabrielsen‡, Ole Kristoffer Olstad§, Nancy Morrison‖, Olav Sæther§, Ola R. Blingsmo§, Vigdis T. Gautvik§, Jan Gordeladze§, Anne Karine Haflan, Edward F. Voelkel‡‡, Tordis B. Øyen‡, Armen H. Tashjian, Jr.‡‡, and Kaare M.Gautvik§‖**

*From the ‡Department of Biochemistry, University of Oslo, P. O. Box 1041 Blindern, 0316 Oslo 3, Norway, the §Institute of Medical Biochemistry and Biotechnology Centre, University of Oslo, P.O. Box 1112 Blindern, 0317 Oslo 3, Norway, the ‖Institute for Surgical Research, The National Hospital, University of Oslo, Pilestredet 32, 0027 Oslo 1, Norway, the **Center for Industrial Research, P. O. Box 124 Blindern, 0314 Oslo 3, Norway, and the ‡‡Laboratory of Toxicology, Harvard School of Public Health and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115*

Human parathyroid hormone (hPTH) is susceptible to proteolytical cleavage both in humans and when expressed as a secretory product in *Escherichia coli* (Høgseth, A., Blingsmo, O. R., Sæther, O., Gautvik, V. T., Holmgren, E., Hartmanis, M., Josephson, S., Gabrielsen, O. S., Gordeladze, J. O., Alestrøm, P., and Gautvik, K. M. (1990) *J. Biol. Chem.* 265, 7338–7344) and *Saccharomyces cerevisiae* (Gabrielsen, O. S., Reppe, S., Sæther, O., Blingsmo, O. R., Sletten, K., Gordeladze, J. O., Høgset, A., Gautvik, V. T., Alestrøm, P., Øyen, T. B., and Gautvik, K. M. (1990) *Gene (Amst.)* 90, 255–262). In the latter system, one major site of cleavage was identified (Arg^{25}-Lys^{26}↓Lys^{27}). To produce hPTH resistant to this proteolytic processing, a point mutation changing Lys^{26} to Gln was introduced, and the modified gene expressed in *S. cerevisiae* as a fusion protein with the α-factor leader sequence. The resulting major form of hPTH secreted to the growth medium was of full length showing that the mutation had eliminated internal processing. Consequently, the yield of the mutant hormone was significantly higher than obtained with the natural peptide. Using improved purification procedures, a significantly higher purity was also obtained. The secreted mutant hPTH-(1–84,Q26) had the correct size, full immunological reactivity with two different hPTH antisera, correct amino acid composition and N-terminal sequence, and correct mass as determined by mass spectrometry. Furthermore, the introduced mutation did not reduce the biological activity of the hormone as judged from its action in three biological assay systems: 1) a hormone-sensitive osteoblast adenylate cyclase assay; 2) an *in vivo* calcium mobilizing assay in rats; and 3) an *in vitro* bone resorption assay.

Gene fusions with the mating pheromone α-factor leader sequence have been widely used to express, correctly process, and efficiently secrete human proteins of medical interest in *Saccharomyces cerevisiae* (1–4). Contrary to the situation in higher eukaryotes where the propeptide processing signal seems to involve proximal residues in addition to the doublet of basic amino acids (5, 6), there is no evidence that yeast yscF endopeptidase (*KEX2* gene product) requires more than a pair of basic amino acids to process a propeptide, although a Lys-Lys pair is a poor substrate for the enzyme (6, 7). Since the probability of finding two consecutive basic amino acids (Lys-Arg, Arg-Lys, or Arg-Arg) in a random protein sequence is rather high, many heterologous proteins risk to be aberrantly processed during secretion in yeast. Thus, in several cases where such sites have been present in the mature peptides, the secretion products were reported to be either partially (8) or completely (9) cleaved, heterogeneous (10), or to contain extra unidentified peptides (11), suggesting aberrant processing.

The mature human parathyroid hormone (hPTH)[1] illustrates this problem because it contains two potential processing sites (Arg-Lys) in the 84-amino acid-long molecule. Hence, when the hPTH gene, fused to the α-factor leader, was expressed in *S. cerevisiae*, a significant fraction of the synthesized protein was cleaved at the first of these sites. The cleavage site was localized after the two basic amino acids (Arg^{25}-Lys^{26}↓Lys^{27}), suggesting that the yscF protease is involved (12).

In the present report we describe the production in yeast of a mutant hPTH where the internal putative yscF cleavage site has been removed by *in vitro* mutagenesis, substituting lysine at position 26 (Lys^{26}) by glutamine (Gln^{26}). As a consequence, the major degradation product hPTH-(27–84) (12) was no longer found in the growth medium while the yield of full-length hormone increased. The secreted hPTH-(1–84,Q26) had correct size, mass, sequence, and immunoreactivity. Furthermore, the introduced mutation did not reduce the biological activity of the hormone as judged from its action in several biological test systems.

* This project has been supported in part by the company Selmer-Sande A/S, by The Royal Norwegian Council for Scientific and Industrial Research Grants PT 15.18471 and PT 15.15449, by the Anders Jahre's Foundation for the Promotion of Science, and by the Rakel and Otto K. Bruun's legacy. The project was also funded by a research grant from the Nordic Yeast Research Program to OSG and a Research Grant DK 10206 from the National Institutes of Health (to A. H. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

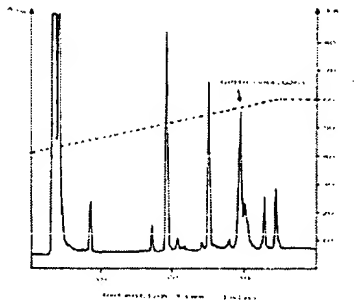
¶ To whom correspondence should be addressed: Dept. of Biochemistry, University of Oslo, P. O.Box 1041 Blindern, 0316 Oslo 3, Norway. Tel.: 47-2-45-61-73; Fax: 47-2-45-41-94.

[1] The abbreviations used are: hPTH, Human parathyroid hormone; hPTH-(1–84), full-length (84 amino acids long) human parathyroid hormone with no mutations; hPTH-(1–84,Q26), full-length human parathyroid hormone where amino acid Lys^{26} is mutated to Gln^{26}; prefix y, produced in yeast (*S. cerevisiae*); prefix s, synthetic (commercial preparation).

FIGURE 1.

SDS PAGE of concentrated yeast growth medium containing mutated and non-mutated hPTH. Aliquots of concentrated growth medium from yeast strain BJ1991 transformed with the expression plasmids priUXPTH-Q36 (lane 3) and priUXPTH-2 (12) (lane 2) were analysed by 15% PAGE in the presence of 0.1% SDS, and visualised by silver staining as described in Materials and Methods.
Lane 4: molecular size markers.
Lane 1: a commercial hPTH standard (Bachem).

FIGURE 2A.



HPLC purification of yeast derived hPTH(1-84,Q26). Yeast medium concentrated on S Sepharose FF was further purified as described in Materials and Methods. The elution profile from the first HPLC step, a Vydac C18 column, is shown. The peak containing yhPTH(1-84,Q26) is marked. Several peaks probably represent non-peptide low molecular weight substances from the fermentation broth since they do not give rise to bands when analysed by SDS-PAGE.

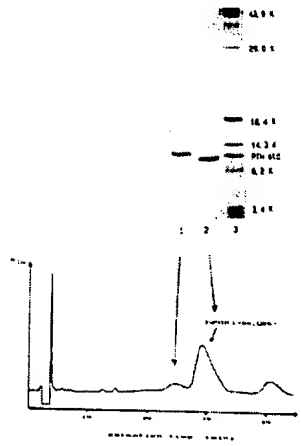


FIGURE 2B.

The elution profile from the second HPLC step, a Waters Nova-Pak C18 column, isocratic elution, with an analysis of the final purity of yhPTH(1-84,Q26) (0.6 mg) as revealed by SDS PAGE and silver staining. Lane 2 shows yhPTH(1-84,Q26) from the main HPLC peak. The molecular weight marker in lane 3 is the same as in Figure 1, but includes a reference hPTH produced in *E.coli*. Lane 1 shows a slower migrating hormone (0.6 mg) which is O-glycosylated (unpublished results), and that elutes in the minor front peak from the second HPLC.

Table 1

N-terminal amino acid sequence of yhPTH(1-84,Q26).

```
                5                  10                  15
Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-
            20                  25                  30
Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Gln-Lys-Leu-Gln-Asp
```

See Materials and Methods for experimental details.

Table 2

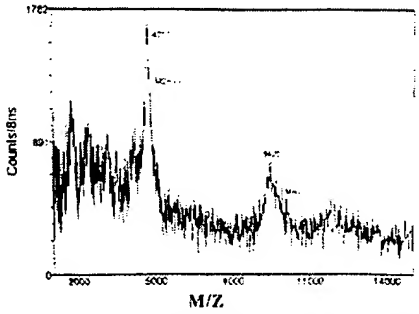
Total amino acid composition of yhPTH(1-84,Q26).

	yhPTH(1-84,Q26)		shPTH(1-84)		shPTH(1-84)[1]	
Aa	Found	Theory	Found	Theory	Found	Theory
Asx	10.4	10	10.3	10	10.4	10
Glx	11.8	12	10.7	11	10.8	11
Ser	7.4	7	7.3	7	7.4	7
Gly	4.6	4	4.4	4	4.5	4
His	3.7	4	3.8	4	3.7	4
Thr	1.2	1	1.2	1	1.1	1
Ala	6.9	7	6.7	7	6.8	7
Arg	5.3	5	5.4	5	5.4	5
Pro	3.3	3	3.2	3	3.0	3
Val	7.5	8	7.8	8	7.7	8
Met	1.8	2	1.8	2	1.7	2
Ile	0.9	1	0.9	1	0.8	1
Leu	9.5	10	9.6	10	9.7	10
Phe	1.2	1	1.1	1	1.1	1
Lys	7.6	8	8.7	9	8.8	9
Trp	nd.	1	nd.	1	nd.	1
Sum	83.1	84	82.9	84	82.9	84

[1] Bachem standard hPTH

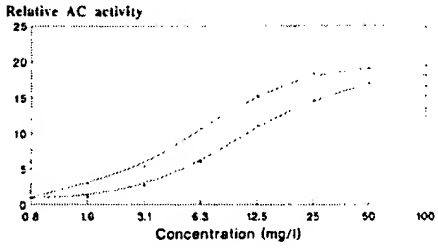
See Materials and Methods for experimental details.

FIGURE 3.



Plasma desorption mass spectrometry analysis of purified recombinant yhPTH(1-84,Q26). The spectrum shows the ion abundance as a function of mass over charge.

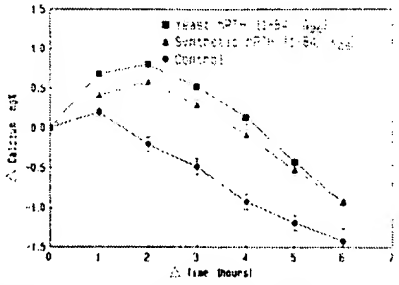
FIGURE 4.



Adenylate cyclase-stimulating acitivity of yhPTH(1-84,Q26)

Purified recombinant hPTH(1-84,Q26) (•-•) was assayed for biological activity in a hormone-sensitive osteoblast adenylate cyclase (AC) assay as described in Materials and Methods. Relative AC activity means relative to control-activity set equal to 1. Each point gives the mean value of triplicate samples. hPTH(1-84) from Bachem (○-○) was used as reference.

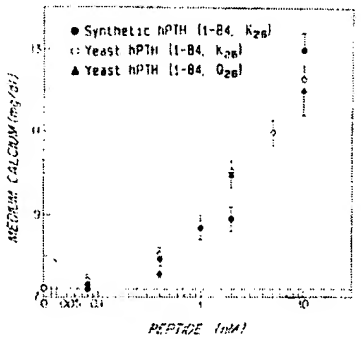
FIGURE 5.



In vivo induction of hypercalcemia by yhPTH(1-84,Q26)

Parathyroidectomized male Wistar rats were tested for induction of hypercalcemia by yhPTH(1-84,Q26) and Bachem shPTH(1-84) standard as described in Materials and Methods. Blood samples were drawn at 0, 1, 2, 3, 4, 5, and 6 hours after injection of PTH (2 μg of yhPTH(1-84,Q26) and 2.75 μg of shPTH(1-84)). The induced hypercalcemia is given as change (Δ) in serum calcium concentration above the zero-time control value.

FIGURE 6.



In vitro bone resorption-stimulating activity of yhPTH(1-84,Q26)

Dose response relationships for shPTH(1-84), yhPTH(1-84), and yhPTH(1-84,Q26) as bone resorption-stimulating agents. Each point gives the mean value and the brackets give the SE for groups of 5 to 28 bones. Results were obtained from up to 5 independent experiments and were pooled where statistically valid. The medium calcium concentration from control bones was 7.3 ± 0.08 mg/dl for 33 separate bones and is shown by the open square and dashed horizontal line. The treatment period was 72 hours.

MATERIALS AND METHODS AND RESULTS[2]

DISCUSSION

The hPTH appears to be especially sensitive to proteolytic degradation. Large amounts of hPTH fragments are found in the parathyroid gland (13), and only about 10% of circulating hPTH immunoreactive peptides are full-length hormone (14, 15). Similarly, degradation of hPTH has been a major problem when the hormone is expressed in heterologous organisms (16, 17). Improved yields of intact hormone have been obtained by secretion of the hormone into the growth medium, either in *Escherichia coli* (18) or in *S. cerevisiae* (12) or by expressing hPTH as an intracellular fusion protein in *E. coli* (19, 20). Even in these systems, proteolytically cleaved forms may be produced reducing the yield of intact hormone.

The present results show that hPTH can be expressed in a correctly processed and secreted in a largely intact form in *S. cerevisiae* after the introduction of a single, structurally conservative mutation in the 26th amino acid ($Lys^{26} \rightarrow Gln$). The hormone produced resists the frequent cleavage found in the non-mutated hormone (12).

The increase in final yield from shake flask cultures of pure full-length hormone agonist is 5–10-fold compared with that obtained with the nonmutated hormone expressed in the same system (12) using identical culture conditions (results not shown). An improved purification procedure gave homogeneous hPTH-(1–84,Q26) after two high performance liquid chromatography steps.

The mutation was introduced in a region of hPTH previously found to be essential for biological activity (13) and within a triplet of basic amino acids conserved between the rat (21), porcine (22), bovine (23), and human hormone (24, 25). However, no difference between the activity of recombinant yhPTH-(1–84,Q26) and standard shPTH-(1–84) could be detected in three different biological assays: an adenylate cyclase assay, measurement of the ability to induce hypercalcemia in parathyroidectomized rats, and the mouse calvarial assay for bone resorption-stimulating activity. It is possible that the dibasic site in hPTH in fact functions as an easily attacked proteolytic site that destabilises the hormone and thus allows more rapid fluctuations in the hormone levels. A more degradation-resistant hPTH agonist could therefore be of potential importance when used as a medical drug due to different pharmacokinetics.

hPTH is one of the primary calcium-regulating hormones in the body and acts principally on kidney and bone cells, stimulating renal calcium reabsorption, phosphate excretion, and bone remodelling, respectively (26–28), resulting in anabolic as well as catabolic effects. Its overall physiological action is probably to generate a positive calcium balance and enhance bone formation. A fragment of hPTH together with 1,25-$(OH)_2$ vitamin D_3 have also been reported to induce bone formation in humans (29, 30), and one of the major areas of potential use of a recombinant hPTH is therefore in the treatment of postmenopausal osteoporosis. To evaluate such applications, sufficient supplies of recombinant hPTH or its agonists are essential.

Acknowledgment—We are grateful to Dr. Knut Sletten, Department of Biochemistry, University of Oslo, Norway, for doing the amino acid sequence and composition analysis.

[2] Portions of this paper (including "Materials and Methods," "Results," Figs. 1–6, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

REFERENCES

1. Brake, A. J., Merryweather, J. P., Coit, D. G., Heberlein, U. A., Masiarz, F. R., Mullenbach, G. T., Urdea, M. S., Valenzuela, P., and Barr, P. J. (1984) *Proc. Natl. Acad. Sci U. S. A.* **81**, 4642–4646
2. Vlasuk, G. P., Bencen, G. H., Scarborough, R. M., Tsai, P.-K., Whang, J. L., Maack, T., Camargo, M. J. F., Kirsher, S. W., and Abraham, J. A. (1986) *J. Biol. Chem.* **261**, 4789–4796
3. Zsebo, K. M., Lu, H.-S., Fieschko, J. C., Goldstein, L., Davis, J., Duker, K., Suggs, S. V., Lai, P.-H., and Bitter, G. (1986) *J. Biol. Chem.* **261**, 5858–5865
4. Loison, G., Findeli, A., Bernard, S., Nguyen-Juilleret, M., Marquet, M., Riehl-Bellon, N., Carvallo, D., Guerra-Santos, L., Brown, S. W., Courtney, M., Roitsch, C., and Lemoine, Y. (1988) *BioTechnology* **6**, 72–77
5. Harris, R. B. (1989) *Arch. Biochem. Biophys.* **275**, 315–333
6. Foster, D. C., Sprecher, C. A., Holly, R. D., Gambee, J. E., Walker, K. M., and Kumar, A. A. (1990) *Biochemistry* **29**, 347–354
7. Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984) *Cell* **37**, 1075–1089
8. Jacobson, M. A., Forma, F. M., Buenaga, R. F., Hofmann, K. J., Schultz, L. D., Gould, R. J., and Friedman, P. A. (1989) *Gene (Amst.)* **85**, 511–516
9. Innis, M. A., Holland, M. J., McCabe, P. C., Cole, G. E., Wittman, V. P., Tal, R., Watt, K. W. K., Gelfand, D. H., Holland, J. P., and Meade, J. H. (1985) *Science* **228**, 21–26
10. Mullenbach, G. T., Tabrizi, A., Blacher, R. W., and Steimer, K. S. (1986) *J. Biol. Chem.* **261**, 719–722
11. Moody, A. J., Norris, F., Norris, K., Hansen, M. T., and Thim, L. (1987) *FEBS Lett.* **212**, 302–306
12. Gabrielsen, O. S., Reppe, S., Sæther, O., Blingsmo, O. R., Sletten, K., Gordeladze, J. O., Høgset, A., Gautvik, V. T., Alestrøm, P., Øyen, T. B., and Gautvik, K. M. (1990) *Gene (Amst.)* **90**, 255–262
13. Cohn, D. V., and Elting, J. (1983) *Recent Prog. Horm. Res.* **39**, 181–209
14. Gautvik, K. M., Gautvik, V. T., and Halvorsen, J. F. (1983b) *Scand. J. Clin. Lab. Invest.* **43**, 553–564
15. Slatopolsky, E., Martin, K., Morrissey, J., and Hruska, K. (1982) *J. Lab. Clin. Med.* **90**, 309–316
16. Born, W., Freeman, M., Hendy, G. N., Rapoport, A., Rich, A., Potts, J. T. J., and Kronenberg, H. M. (1987) *Mol. Endocrinol.* **1**, 5–14
17. Rabbani, S. A., Yasuda, T., Bennett, H. P. J., Sung, W. L., Zahab, D. M., Tam, C. S., Goltzman, D., and Hendy, G. N. (1988) *J. Biol. Chem.* **263**, 1307–1313
18. Høgset, A., Blingsmo, O. R., Sæther, O., Gautvik, V. T., Holmgren, E., Hartmanis, M., Josephson, S., Gabrielsen, O. S., Gordeladze, J. O., Alestrøm, P., and Gautvik, K. M. (1990) *J. Biol. Chem.* **265**, 7338–7344
19. Wingender, E., Bercz, G., Blöcker, H., Frank, R., and Mayer, H. (1989) *J. Biol. Chem.* **264**, 4367–4373
20. Gardella, T. J., Rubin, D., Abousamra, A. B., Keutmann, H. T., Potts, J. T., Kronenberg, H. M., and Nussbaum, S. R. (1990) *J. Biol. Chem.* **265**, 15854–15859
21. Heinrich, G., Kronenberg, H. M., Potts, J. T., Jr., and Habener, J. F. (1984) *J. Biol. Chem.* **259**, 3320–3329
22. Sauer, R. T., Niall, H. D., Hogan, M. L., Keutmann, H. T., O'Riordan, J. L. H., and Potts, J. T. J. (1974) *Biochemistry* **13**, 1994–1999
23. Kemper, B., Habener, J. F., Potts, J. T., Jr., and Rich, A. (1976) *Biochemistry* **15**, 20–25
24. Keutmann, H. T., Sauer, M. M., Hendy, G. N., O'Riordan, J. L. H., and Potts, J. T. J. (1978) *Biochemistry* **17**, 5723–5729
25. Vasicek, T. J., McDevitt, B. E., Freeman, M. W., Fennick, B. J., Hendy, G. N., Potts, J. T., Jr., Rich, A., and Kronenberg, H. M. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 2127–2131
26. Norman, A. W., Roth, J., and Orci, L. (1982) *Endocr. Rev.* **3**, 331–366
27. Morel, F. (1983) *Recent Prog. Horm. Res.* **39**, 271–304
28. Potts, J. T. J., Kronenberg, H. M., and Rosenblatt, M. (1982) *Adv. Protein Chem.* **35**, 323–396
29. Reeve, J., Meunier, P. J., Parsons, J. A., Bernat, M., Bijvoet, O. L. M., Courpron, P., Edouard, C., Klenerman, L., Neer, R. M., Renier, J. C., Slovik, D., Vismans, F. J. F. E., and Potts, J. T. Jr. (1980) *Br. Med. J.* **280**, 1340–1351
30. Slovik, D. M., Rosenthal, D. I., Doppelt, S. H., Potts, J. T. Jr.,

Daly, M. A., Campbell, J. A., and Neer, R. M. (1986) *J. Bone Miner. Res.* **1**, 377–381
31. Carter, B. L. A., Irani, M., Mackay, V. L., Seale, R. L., Sledziewski, A. V., and Smith, R. A. (1987) in *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. III, pp. 141–161, IRL Press, Oxford
32. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
33. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
34. Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K., and Davis, R. W. (1979) *Gene (Amst.)* **8**, 17–24
35. Gautvik, K. M., Teig, V., Halvorsen, J. F., Arnesen, E., Myhre, L., Heimann, P., and Tollman, R. (1979) *Scand. J. Clin. Lab. Invest.* **39**, 469–478
36. Laemmli, U. K. (1970) *Nature* **227**, 680–685
37. Bürk, R. R., Eschenbruch, M., Leuthard, P., and Steck, G. (1983) *Methods Enzymol.* **91**, 247–254
38. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
39. Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
40. Gautvik, K. M., Gordeladze, J. O., Jahnsen, T., Haug, E., Hansson, V., and Lystad, E. (1983) *J. Biol. Chem.* **258**, 10304–10311
41. Gautvik, K. M., Gordeladze, J. O., Moxheim, E., and Gautvik, V. T. (1984) *Eur. Surg. Res.* **16**, 41–54
42. Robinson, D. R., Tashjian, A. H., and Levine, L. (1975) *J. Clin. Invest.* **56**, 1181–1188
43. Ivey, J. L., Wright, D. R., and Tashjian, A. H. (1976) *J. Clin. Invest.* **58**, 1327–1338

Supplemental material

to

Characterization of a K26Q site directed mutant of human parathyroid hormone expressed in yeast

by

Sjur Reppe, Odd S. Gabrielsen, Ole Kristoffer Olstad, Nancy Morrisen, Olav Sæther, Ola R. Blingsmo, Vigdis T. Gautvik, Jan Gordeladze, Anne Karine Haflan, Edward F. Voelkel, Tordis B. Øyen, Armen H. Tashjian Jr., and Kaare M.Gautvik

MATERIALS AND METHODS

Plasmids, strains and culture conditions.

The *Saccharomyces cerevisiae* strain used for hPTH expression was BJ1991 (a, *trp1, ura3-52, leu2, prb1-1122, pep4-3*). Yeast cells were transformed by the lithium method (31), and transformants grown at 30°C in YNBGC medium (0.67% yeast nitrogen base, 2% glucose, 1% casamino acids (Difco)). The pαUXPTH-2 plasmid used as a reference for expression of authentic hPTH(1-84) in yeast, and the subclone M13PTH-3, have been described previously (12; US Patent application no.921684).

In vitro mutagenesis of the α-factor hPTH fusion gene.

The inappropriate processing of hPTH secreted from yeast (cleaved at the site: Arg25.Lys26↓Lys27 (12)), might be eliminated by a change from Lys26 to Glu26 to destroy the potential *yscF* processing site. To introduce this change, the subclone M13PTH-3 was modified by *in vitro* mutagenesis using the "Muta-gene™ in vitro mutagenesis kit" (Bio-Rad) based on the method of Kunkel *et al.*, (32) and the mutagenizing oligoeucleotide: 5'-GGCTGCCTCAGAAGCTGC-3'. Positive plaques (containing M13PTH-Q26) were verified by DNA sequencing (33)..The entire expression cassette between a *Bam*HI and a filled-in *Eco*RI site was isolated from M13PTH-Q26 and inserted between the *Bam*HI and *Pvu*II site of the yeast shuttle vector YEp24 (34). This expression plasmid was designated pαUXPTH-Q26 and used to transform the yeast strain BJ1991.

Detection of hPTH in culture media.

Radioimmunoassay of hPTH in yeast culture media was performed as described (12,35). For electrophoretic analysis, yeast culture media were concentrated on S Sepharose FF as previously described (12), and separated on a 15% polyacrylamide gel in the presence of SDS (36), and either stained with silver (37) or analysed further by protein blotting using Immobilon PVDF Transfer Membranes (Millipore) and the buffers of Towbin *et al* (38). Reference hPTH(1-84) was purchased from Peninsula Laboratories (USA) and Sigma (USA). Protein blots were visualised as described (12).

Purification of hPTH from yeast culture medium.

The clarified culture medium was concentrated on a S Sepharose Fast Flow column previously equilibrated with 0.3M glycine, pH 3.0. The medium was adjusted to pH 3.0 with 1 M HCl before loading. The column was then washed with 0.1M acetic acid (adjusted to pH 6.0) and eluted with 0.1M Na_2HPO_4, pH 8.5. hPTH coeluted with the A_{280} peak. This step also resulted in a partial purification of the hormone. Final purification was obtained by a two-step reverse phase HPLC procedure. In the first step a Vydac protein/peptide C18 column (250x4.6 mm, purchased from "The separation group", Hesperia, CA,USA) was employed with a gradient from 40-60% eluant B (eluant A: 0.2% trifluoroacetic acid in milliQ water, and eluant B: 0.2% trifluoroacetic acid in 70% acetonitrile and 30% milliQ water). The flow rate was 1 ml/min. In the second step, a Waters Nova-Pak C18 column (10.0x0.8 cm) was used with isocratic elution using 47% eluant B. Both eluates were monitored by UV at 220 nm.

N-terminal sequence, amino acid composition and mass spectrometry

Proteins to be sequenced were purified either by HPLC as described above or by SDS polyacrylamide gelelectrophoresis followed by blotting onto Immobilon PVDF membranes (39). Automated Edman degradation was performed on a 477A Protein Sequencer with an on-line 120A phenylthiohydantoin amino acid analyser from Applied Biosystems (Foster City, CA, USA). All reagents were obtained from Applied Biosystems. The amino acid composition was analysed on a Biotronic SP4100 amino acid analyzer after hydrolysis of 30 µg of the hPTH peptide in 6M HCl (special purity), 10.05% thioglycol acid, for 24 hr at 110 °C.

The molecular mass of purified recombinant hPTH(1-84,Q26) was determined by ^{252}Cf plasma desorption mass spectrometry as described (18), and was kindly performed by Dr. Maria Hartmanis, KabiGen AB, Stockholm.

Adenylate cyclase assay for hPTH.

The adenylate cyclase stimulating activity of the recombinant hPTH was assayed as previously described using UMR 106 osteosarcoma cell membranes (12,40,41). Synthetic hPTH(1-84) from Sigma was used as reference standard.

Assay of hPTH-induced hypercalcemia in rats

Male Wistar rats (150-200 g) were parathyroidectomized using electrocautery 18 hours prior to the start of the experiment. The animals were fasted overnight, and anesthetized the next day using Hypnorm Dormicum (0.2 ml/rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers Acetate, 4% bovine albumin, 25 Units heparin/ml. Five minutes after injection of 200 µl of the heparinized Ringers, a baseline blood sample was drawn (300 µl). The animals were tracheotomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. PTH was then injected subcutaneously in the neck in a volume of 200 µl. Both hPTH(1-84) and hPTH(1-84,Q26) were dissolved in 100 µl 0.01N acetic acid. After dissolving, the solutions were brought up in 900 µl of Ringers Acetate containing 1% bovine albumin. Blood samples were drawn from the carotid artery at 1, 2, 3, 4, 5, and 6 hours after the injection of PTH. The rats were re-heparinized 5 minutes before drawing each blood sample using 200 µl of the heparinized Ringers solution.The concentrations of PTH used were determined by amino acid analysis. hPTH(1-84,Q26) was used at 2 µg/animal, and the Bachem reference PTH was used at 2.75 µg/animal (a higher numerical value used to compensate for a discrepancy between what was labelled on the Bachem vial (1 mg PTH), and what we determined by amino acid analysis (0.75 mg PTH)). The blood samples were centrifuged for 10 minutes, and the plasma was analysed for calcium using a Cobas Autoanalyzer (Cobas Bio, Roche Diagnostics).

Bone resorption-stimulating activity

Bone resorption-stimulating activity was measured using neonatal mouse calvaria as previously described (42,43). The medium was Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated (56-60°C for 30 min) horse serum. Incubation of calvaria from 4- to 5-day-old mice was performed at 37°C in a roller drum apparatus under an atmosphere of 50% O_2, 5% CO_2, and 45% N_2. Calvariae were preincubated for 24 hours before experimental treatment was begun. The medium was then changed, and fresh control medium or medium containing standard synthetic PTH [shPTH(1-84)], yeast derived hormone [yhPTH(1-84)], or yeast derived mutant hormone [yhPTH(1-84,Q26)] was added. Bone resorption was determined by measuring the accumulation of $^{45}Ca^{2+}$ in the culture medium during the 72-hour treatment period. The concentration of total calcium in the bone culture medium was measured in 400 µl samples by a calcium-selective electrode using a Nova 7+7 automatic calcium analyzer (NOVA Biomedical, Waltham, MA, USA). Results in each experiment were subjected to an analysis of variance, and the SE values were calculated from the residual error term of that analysis. Experiments were pooled when the variances were nonheterogeneous. The shPTH(1-84) standard was from Bachem (Lot no. 070589).

RESULTS

Expression and secretion of hPTH(1-84,Q26) in yeast.

To prevent the internal processing of hPTH occuring during secretion in yeast, a point mutation was introduced in codon 26 of the hPTH gene changing lysine-26 to glutamine in the peptide. The mutated gene was expressed in *Saccharomyces cerevisiae* after fusion to the α-factor prepro-leader sequence and promoter. The products secreted to the growth medium were analysed by SDS-PAGE (Fig.1). In the non-mutated control (lane 2) the strongest band had a molecular mass well below the standard hPTH, and microsequencing showed that it corresponded to the hormone fragment hPTH(27-84) (12). After mutation (lane 3), this band was absent, showing that the cleavage between amino acid 26 and 27 was eliminated as a result of the introduced mutation. The major product was a polypeptide that migrated close to the full length hPTH standard, slightly faster in an anionic gel system (Fig.1) and slightly slower in a cationic gel system (not shown), in accord with the single charge difference between the mutated and the wild type proteins. In addition to the main product, several other bands were present of apparently higher molecular mass which appear to be O-glycosylated forms of yhPTH(1-84,Q26)(unpublished experiments).The yhPTH(1-84,Q26) product also migrated close to the hPTH standard when analyzed by two dimensional gel electrophoresis (first dimension acetic acid/urea gel, second dimension SDS polyacrylamide gel, not shown) and, by protein blotting, it was found to react with two hPTH specific antibodies against the 1-34 amino acid and 44-68 amino acid respectively (not shown), supporting that we are dealing with a full length hormone.

Purification

To purify yhPTH(1-84,Q26), growth medium was first concentrated using a S Sepharose FF column (yielding 70% pure yhPTH(1-84,Q26)) (Fig.1). The hPTH peak from the first HPLC step contained predominantly yhPTH(1-84,Q26), but also some glycosylated hormone with reduced migration in SDS PAGE (Fig.3A). After a second isocratic HPLC chromatography, only a single band was seen after SDS PAGE (Fig.2B). When the yield from shake-flask cultures of pure yhPTH(1-84,Q26) was compared with that of yhPTH(1-84), 5-10 fold higher yields were obtained, consistent with our previous estimate of the fraction of full length hormone (up to 20%) produced with an expression plasmid encoding non-mutated hPTH(1-84) (12).

Biochemical characterization of yhPTH(1-84,Q26)

The nature of the yhPTH(1-84,Q26) product was verified by N-terminal amino acid sequencing showing correct amino-terminal sequence (Table 1). In particular, the expected change from Lys to Gln in position 26 was confirmed. The amino acid composition of the purified yhPTH(1-84,Q26) is shown in Table 2. Except for one Trp that is not revealed under the conditions used, the expected composition and total number of amino acid residues were found. Therefore, no C-terminal degradation had occurred. To substantiate that the purified yhPTH(1-84,Q26) represented the intact hormone, mass spectrometry was performed giving a plasma desorption mass spectrum as shown in Fig.3. A molecular mass of 9420±9 daltons could be calculated from the single-charged and double-charged molecular ions present in the spectrum. This value corresponds well with the theoretical molecular mass of hPTH(1-84,Q26) calculated from the amino acid composition, of 9418.

Biological characterization

The biological activity of the yhPTH(1-84,Q26) was tested by three different assays. The purified yhPTH(1-84,Q26) was first analysed for its ability to stimulate the adenylate cyclase activity in rat UMR 106 osteosarcoma cells (40,41) (Fig.4). The stimulation curve coincides with that of purified non-mutated yhPTH(1-84). In this acute assay, no significant difference in biological activity was detected between the native hormone and the degradation-resistant mutated hormone.

Secondly, the purified yhPTH(1-84,Q26) and a standard shPTH(1-84) were assayed for the ability to induce hypercalcemia in parathyroidectomized rats (Fig.5). The yhPTH(1-84,Q26) had an *in vivo* biological activity stimulatory effect comparable to or slightly above that of the synthetic hPTH control.

Finally, the yhPTH(1-84,Q26) was tested for direct activity on bone by assaying its bone resorption-stimulating activity using neonatal mouse calvaria. yhPTH(1-84,Q26) stimulated bone resorption *in vitro* with a potency similar to that of shPTH(1-84) and yhPTH(1-84) (Fig.6). A time-course experiment similarly showed that yhPTH(1-84,Q26) had the same bone resorption-stimulating activity as the non-mutated hormone (not shown). Thus, we have found no indication of reduced biological activity resulting from the introduced mutation.